

Regulation of pancreatic cancer cells by suppressing KIN17 through the PI3K/AKT/mTOR signaling pathway

QIUYAN LI^{1*}, YUXIA YANG^{1*}, XIAOCONG LIN^{2*}, LOK TING CHU², HELIAN CHEN¹,
LINSONG CHEN¹, JINJING TANG¹ and TAO ZENG¹

¹Department of Medical Laboratory, Affiliated Hospital of Guangdong Medical University, Zhanjiang, Guangdong 524000, P.R. China;

²Institute of Biochemistry and Molecular Biology, Guangdong Medical University, Zhanjiang, Guangdong 524023, P.R. China

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Abstract. Pancreatic cancer is an aggressive tumor, which is often associated with a poor clinical prognosis and resistance to conventional chemotherapy. Therefore, there is a need to identify new therapeutic markers for pancreatic cancer. Although KIN17 is a highly expressed DNA- and RNA-binding protein in a number of types of human cancer, its role in pancreatic cancer development, especially in relation to progression, is currently unknown. The present study verified the upregulation of KIN17 in pancreatic cancer using The Cancer Genome Atlas and Gene Expression Omnibus databases (GSE15471, GSE71989 and GSE62165), and identified an association between the PI3K/Akt/mTOR pathway and patient prognosis using publicly available datasets (Gene Expression Profiling Interactive Analysis). Immunohistochemistry was performed to determine the association between KIN17 and the pathological features of clinical pancreatic cancer samples. Furthermore, knockdown of KIN17 was shown to inhibit the migration and invasion of pancreatic cancer cells, and to reverse epithelial-mesenchymal transition in pancreatic cancer cells through downregulation of Vimentin and N-cadherin, and upregulation of E-cadherin. Through various cellular experiments, the role of KIN17 was explored in PI3K/AKT/mTOR activity. KIN17 inhibition was shown to suppress the migration and invasion of pancreatic cancer cells through PI3K/AKT/mTOR-mediated autophagy.

Furthermore, combined with mTOR inhibition, dual inhibition could enhance autophagy, leading to anti-migratory and anti-invasion effects in pancreatic cancer. In conclusion, the present study indicated that KIN17 may have a role in carcinogenesis and could serve as a prognostic biomarker of pancreatic cancer, owing to its high expression. In addition, KIN17 may be considered a potential therapeutic target with its knockdown having an inhibitory effect on pancreatic cancer.

Introduction

Pancreatic cancer is a highly malignant type of cancer with a high metastatic rate. With the abundance of pancreatic blood and lymphatic vessels, pancreatic cancer rapidly grows; this also results in early invasion and metastasis, which are associated with a poor prognosis, and eventually mortality (1). Notably, pancreatic cancer is difficult to diagnose in the early stages; therefore, it often metastasizes and is resistant to drug therapy, thus making it difficult to reduce the high mortality rate (2). Therefore, it is important to identify biomarkers capable of early diagnosis and an appropriate therapeutic target for pancreatic cancer.

The prognosis of patients with pancreatic cancer largely depends on the cancer stage at the time of diagnosis. CA19-9 is a widely used biomarker for pancreatic cancer prognosis; however, its sensitivity and specificity are insufficient (3). The main challenge in improving the recovery rate of patients with pancreatic cancer is the lack of biomarkers for early detection.

KIN17 is a highly conserved gene in mammalian cells containing an N-terminal zinc finger structure (27-50 nucleotides) and a C-terminal KOW membrane (335-373 nucleotides), which is located on human chromosome 10p15-p14 (4). KIN17 has been reported to participate in cellular activities and physiological processes, including DNA replication, RNA transcription and cell cycle regulation (5). Recently, KIN17 has been established as having an essential role in various types of cancer. Notably, Dai *et al* (6) and Huang *et al* (7) demonstrated that KIN17 may serve an important role in promoting epithelial-mesenchymal transition (EMT) in hepatocellular carcinoma and luminal-A breast cancer.

Cancer metastasis, which is a main cause of cancer-related death, can exacerbate the progression of tumor development (8). EMT refers to the phenotypic changes produced by

Correspondence to: Professor Tao Zeng, Department of Medical Laboratory, Affiliated Hospital of Guangdong Medical University, 57 Renmin Avenue South, Xiashan, Zhanjiang, Guangdong 524000, P.R. China
E-mail: zengt@smu.edu.cn

*Contributed equally

Abbreviations: EMT, epithelial-mesenchymal transition; qPCR, quantitative PCR; IHC, immunohistochemistry; 3-MA, 3-methyladenine; CQ, chloroquine; GEO, Gene Expression Omnibus; WB, western blotting

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epithelial cells during a specific process of transformation into stromal cells, thereby endowing them with increased invasiveness, which is a major factor in the metastatic ability and drug resistance of cancer (9,10). Autophagy is a mechanism of membrane-mediated degradation and recovery that is crucial for cellular homeostasis, which can be significantly upregulated by various physiological stimuli, such as malnutrition, hypoxia, endoplasmic reticulum stress, and immune and hormonal stimulation (11). Autophagy serves a dual role in cancer progression because it can hinder or promote cancer occurrence and development (12). Although the mechanism underlying the progression of autophagy is complex, the PI3K/AKT/mTOR pathway has a role in suppressing autophagy progression (13). Wei *et al.* (14) demonstrated that arenobufagin may enhance autophagy and cell apoptosis through PI3K/AKT/mTOR pathway inhibition, effectively suppressing the growth of pancreatic cancer cells both *in vitro* and *in vivo*. Moreover, Qian *et al.* (15) reported that the Qingyihuaji Formula could promote apoptosis and pancreatic cancer autophagy by suppressing two signaling pathways: MAPK/ERK and PI3K/AKT/mTOR. However, the role of KIN17 in these pathways has not been studied in depth. Based on the aforementioned findings, it was hypothesized that KIN17 may act as a regulator of the PI3K/AKT/mTOR signaling pathway.

The present study systematically assessed KIN17 expression in clinical pancreatic cancer samples, and revealed how KIN17 expression may affect the clinicopathological characteristics and survival rates of patients. The study explored the functions of KIN17 in terms of cell migration, invasion and autophagy in pancreatic cancer. In summary, the present study highlights novel findings regarding the effects of KIN17 on autophagy through the PI3K/AKT/mTOR pathway, thus affecting the migration and invasion of pancreatic cancer cells.

Materials and methods

Cell lines and cell culture. The human pancreatic cancer cell lines ASPC-1, PANC-1, PACA-2 and BxPC-3 were purchased from Shanghai Gaining Biological Technology Co., Ltd. PANC-1 and PACA-2 cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (PS; Beyotime Institute of Biotechnology), while ASPC-1 and BxPC-3 cells were cultured in 1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS and 1% PS. The normal human pancreatic cell line, HPNE, was obtained from iCell Bioscience, Inc. HPNE cells were cultured in cell culture medium [DMEM: 70%; M3:BaseF (cat. no. M300F-500; Incell Corporation LLC)]; 20%; FBS: 10%) with 1% penicillin-streptomycin (cat. no. 15140-122; Gibco; Thermo Fisher Scientific, Inc.). All cells were incubated at 37°C in an atmosphere containing 5% CO₂. For drug intervention, PANC-1 and PACA-2 cells were treated with autophagy inhibitors or an autophagy activator purchased from MedChemExpress, including 2 mM 3-methyladenine (3-MA; cat. no. (HY-19312), 10 μ M chloroquine (chloroquine; cat. no. HY-17589A) and 100 nM Rapamycin (Rapa; cat. no. HY-10219). The PANC-1

and PACA-2 cells were incubated with the inhibitors/activator for 24 h at 37°C.

Bioinformatics analysis. The Gene Expression Profiling Interactive Analysis (GEPIA) server (<http://gepia.cancer-pku.cn/>) was used to obtain and analyze KIN17 expression data from pancreatic cancer and normal tissues based on The Cancer Genome Atlas (TCGA) and Gene Type Tissue Expression projects, including 179 cancer and 171 normal samples. In addition, expression data were downloaded and extracted from three Gene Expression Omnibus (GEO; <https://www.ncbi.nlm.nih.gov/geo/>) datasets [GSE15471 (16,17), GSE71989 (18) and GSE62165 (19)], which compared the mRNA expression between normal and pancreatic cancer tissues. Raw data were preprocessed in R language (version 4.3.1; <https://www.r-project.org/>). The expression matrix data of GSE15471, GSE71989 and GSE62165 were obtained by probe transformation according to the annotation information of the chip platform, from which the expression value of KIN17 was extracted. Correlation analysis of KIN17 expression and the expression of genes in the Akt/mTOR pathway was performed using TCGA-PAAD (pancreatic adenocarcinoma) data on the GEPIA server. The Kaplan-Meier method was used to draw survival curves using the 'survival' (<https://cran.r-project.org/package=survival>) and 'survminer' (<https://cran.r-project.org/package=survminer>) packages in R software version 4.3.1 (<https://www.r-project.org/>).

Patient samples and immunohistochemistry (IHC). A total of 72 pairs of pancreatic cancer and normal pancreatic tissue samples (mean patient age: 58 years; age range: 33-77 years) were provided by Shanghai Outdo Biotech Co., Ltd. The clinical and pathological characteristics of the patients are shown in Table I. Firstly, the tissue array was placed in an oven, and baked at 63°C for 1 h, after which dewaxing was completed in an automatic staining machine (LEICAST5020; Leica Microsystems GmbH). The slide was placed in an antigen repair instrument (PT Link; Dako; Agilent Technologies, Inc.), and antigen repair was initiated by selecting the program. After repair, the slides were placed in distilled water at room temperature and allowed to cool naturally for >10 min. Subsequently, the slides were rinsed in PBS, a diluted primary antibody (KIN17; 1:200; cat. no. sc-32769; Santa Cruz Biotechnology, Inc.) working solution was added, and the slides were incubated overnight at 4°C. The next day, the slides were warmed at room temperature for 45 min, washed with PBS, and put into an automatic immunohistochemical staining system instrument (Autostainer Link 48; Dako; Agilent Technologies, Inc.); the corresponding programs were selected for blocking (with 3% hydrogen peroxide, 10 min), secondary antibody incubation (10 min) [EnVision™ FLEX+, Mouse, High pH, (Link); cat. no. K8002; Dako; Agilent Technologies, Inc.) and DAB color development according to the manufacturer's protocol at room temperature. Subsequently, hematoxylin staining was performed for 1 min at room temperature. The slides were immersed in 0.25% hydrochloric acid and alcohol (400 ml 70% alcohol + 1 ml concentrated hydrochloric acid) for ~10 sec and were then rinsed with tap water for 5 min. Finally the slides were dried at room temperature and sealed with neutral resin. The tissue array was examined using an

Table I. Association of KIN17 expression with clinicopathological features in pancreatic cancer.

Characteristic	Total (n=72)	Expression of KIN17		χ^2	P-value
		Low	High		
Age				2.217	0.136
<60 years	37	18	19		
≥60 years	35	11	24		
Sex				0.002	0.968
Male	42	17	25		
Female	30	12	18		
Tumor size				0.15	0.698
≤2 cm	14	5	9		
>2 cm	58	24	34		
Differentiation				0.062	0.803
Poor	31	13	18		
Well/Moderate	41	16	25		
TNM stage				0.02	0.888
I and II	39	16	23		
III and IV	33	13	20		
T stage				0.062	0.803
T1, 2	31	13	18		
T3, 4	41	16	25		
Lymph node metastasis				5.957	0.015 ^a
N0	23	14	9		
N1 + pN2	49	15	34		
Distant metastasis				0.018	0.892
M0	49	20	29		
M1	23	9	14		
Vascular invasion				1.952	0.162
No	40	19	21		
Yes	32	10	22		
Perineural invasion				1.245	0.265
No	31	13	18		
Yes	41	16	25		

Statistical analyses were performed using the χ^2 test. ^aP<0.05. TNM, Tumor-Node-Metastasis.

Aperio scanner (Aperio ScanScope XT; Leica Microsystems GmbH). The tissues underwent IHC, and KIN17 staining intensity [classified into four levels, from 0 (negative) to 4 (strong)] and percentage of positive cells (0-100%) was determined. Finally, the staining results were scored by multiplying the intensity level and percentage, and labelled as the rapid (Q) score. The median Q-score (Q=100) served as the cut-off value to classify the patients into low (Q≤100) or high (Q>100) KIN17 expression groups.

Small interfering RNA (siRNA) transfection. The siRNA oligonucleotides specifically designed for KIN17 (siKIN17) and the negative control siRNA (siNC) were purchased from Suzhou GenePharma Co., Ltd. with the following sequences: siNC, sense 5'-UUCUCCGAACGUGUCACGUTT-3', antisense 5'-ACGUGACACGUUCGGAGAATT-3'; siKIN17#1,

sense 5'-GCAGAAGCUACGCUGGUAUTT-3', antisense 5'-AUACCAGCGUAGCUUCUGCTT-3'; siKIN17#2, sense 5'-GGAAUUCGAAAUGACUUUTT-3', antisense 5'-AAAGUCAUUUCGGAAUUCCTT-3'; siKIN17#3, sense 5'-GCAACAUCUCCAAGUCAATT-3', antisense 5'-UUGACUUGGAGAUGUUGCTT-3'. PANC-1 and PACA-2 cells at 70% confluence were transfected with siRNAs (150 pmol) using siRNA-mate (Suzhou GenePharma Co., Ltd.) according to the manufacturer's protocol at room temperature. After 48 and 72 h, the cells were collected for reverse transcription-quantitative PCR (RT-qPCR) and western blotting (WB) to evaluate the corresponding mRNA and protein expression levels, respectively.

RNA extraction and RT-qPCR. Total RNA was extracted from the cultured cells using TRIzol® reagent (Invitrogen; Thermo

Fisher Scientific, Inc.) according to the manufacturer's instructions. RNA quality, including concentration and purity, was evaluated using a NanoDrop Spectrophotometer (NanoDrop; Thermo Fisher Scientific, Inc.). Subsequently, cDNA was obtained from 1 μ g RNA through RT using PrimeScript™ RT Reagent Kit (cat. no. RR047Q; Takara Biotechnology Co., Ltd.). Finally, mRNA expression was examined using qPCR with an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) and using TB Green® Premix Ex Taq™ (Tli RNaseH Plus) (cat. no. RR420A; Takara Biotechnology Co., Ltd.) according to the manufacturer's protocol. The qPCR thermal cycling conditions were as follows: Initial denaturation at 95°C for 30 sec; followed by 60 cycles of denaturation at 95°C for 5 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec. KIN17 expression was normalized to GAPDH, using the $2^{-\Delta\Delta C_q}$ method (20). The primer sequences used were as follows: human KIN17, forward AGACGCTTTGGCACT AAAAGG and reverse AGTGGCATTCATGTGGATGTG; human GAPDH, forward GCACCGTCAAGGCTGAGAAC and reverse TGGTGAAGACGCCAGTGGA.

WB. Protein was extracted from pancreatic cancer cells using RIPA buffer (Beyotime Institute of Biotechnology) and 20 μ g proteins were separated by SDS-PAGE on 8-15% gels, before being transferred to PVDF membranes (MilliporeSigma). After blocking the membranes in 5% milk for 60 min at room temperature, they were incubated with the following primary antibodies: GAPDH (cat. no. 60004-1-Ig; 1:20,000), Vimentin (cat. no. 60330-1-Ig; 1:50,000), Beclin1 (cat. no. 66665-1-Ig; 1:2,000), mTOR (cat. no. 28273-1-AP; 1:50,000), phosphorylated (P)-mTOR (cat. no. 67778-1-Ig; 1:5,000) (all from Proteintech Group, Inc.), KIN17 (cat. no. sc-32769; 1:1,000; Santa Cruz Biotechnology, Inc.), E-cadherin (cat. no. ab40772; 1:10,000), N-cadherin (cat. no. ab76011; 1:10,000), P62 (cat. no. ab109012; 1:10,000), ULK1 (cat. no. ab177472; 1:10,000), PI3K (cat. no. ab40776; 1:5,000) (all from Abcam), LC3B (cat. no. 2775; 1:1,000), AKT (cat. no. 4691; 1:1,000), P-AKT (cat. no. 4060; 1:1,000) and P-ULK1 (cat. no. 6888; 1:1,000) (all from Cell Signaling Technology, Inc.), and P-PI3K (cat. no. AF5905 1:500; Beyotime Institute of Biotechnology) overnight at 4°C. The membranes were then washed with 1X TBS-0.1% Tween-20 and incubated with secondary antibodies for 1 h at room temperature. The following secondary antibodies were used: Anti-rabbit IgG, HRP-linked antibody (1:5,000; cat. no. SA-00001-2; Proteintech Group, Inc.) and anti-mouse IgG, HRP-linked Antibody (1:5,000; cat. no. SA-00001-1; Proteintech Group, Inc.). Finally, bands were visualized using WesternLumaxLight Superior HRP substrate (cat. no. 310209; Zeta-Life) and visualized using a Tanon 5200 imaging system (Tanon Science and Technology Co., Ltd.). The intensity of the protein bands was semi-quantified using Image Lab software (5.2.1) and the protein expression levels were normalized to the respective GAPDH bands. All WB experiments were conducted in triplicate.

Cell migration and invasion assays. During the logarithmic growth phase, 5×10^4 cells/well in serum-free medium were plated in the upper chamber of a 8- μ m Transwell system at room temperature, while 0.6 ml medium supplemented with

10% FBS was added to the lower chamber in a 24-well plate. After incubation at 37°C for 24 h, the translocated cells were fixed with 4% paraformaldehyde for 20 min, followed by staining with 0.1% crystal violet solution at room temperature for 30 min at room temperature. For quantification, images from five random fields were captured and the cells were counted under optical microscope (magnification, x200). In the invasion assay, the upper Transwell chamber was precoated with 1:8 diluted Matrigel (BD Biosciences) at 37°C for 1 h, and the cells were cultured at 37°C incubator for 72 h, whereas the remaining steps were the same as those performed in the migration assay. Each experiment was conducted in triplicate.

Wound-healing assay. A total of 5×10^5 cells/well in a 6-well plate were cultured without 10% FBS. When the cell fusion rate reached 90%, a wound was generated using a 200- μ l pipette tip to draw a straight line at the bottom of the plate. Images of the cells were captured using an optical microscope at 0 h, when the wound was created, and at 24 h. Relative migration was calculated as follows: Relative migration rate = area (0-24 h)/area at 0 h. Each experiment was repeated three times.

Autophagy assay. Autophagy was detected using a CYTO-ID® Autophagy Detection Kit (cat. no. ENZ-KIT175-0050; Enzo Life Sciences, Inc.). After cultivating the cells on a 14x14 mm confocal dish, the culture medium was carefully removed when the cells reached a fusion level of 50-70%. Subsequently, the cells were washed with 100 μ l 1X Assay Buffer, and were incubated with CYTO-ID Green Detection Reagent 2 for 30 min at room temperature. Finally, the stained cells were analyzed using a confocal microscope.

GFP-mRFP-LC3 staining. The mRFP-GFP-LC3 lentiviral vector was purchased from Suzhou GenePharma Co., Ltd., and PANC-1 and PACA-2 cells were infected according to the manufacturer's instructions. The cells stably expressing mRFP-GFP-LC3 were selected by puromycin (1 μ g/ml) and 1×10^4 stably expressing mGFP-RFP-LC3 cells/dish were seeded into a confocal dish. After incubation for 20 h at room temperature, the cells were transfected with siNC or siKIN17 as aforementioned. The autophagosomes were labeled yellow (mRFP and GFP) whereas autolysosomes were labeled red (mRFP only, and the results in five independent fields were observed under a confocal laser-scanning microscope (Olympus Corporation).

Statistical analysis. To ensure accuracy, all experiments were independently repeated three times. Statistical analysis was performed using either SPSS 27.0 statistical software (IBM Corporation) or GraphPad Prism 8 software (Dotmatics). To analyze the significant differences between two groups, a paired Student's t-test was conducted for paired data, while an unpaired Student's t-test was used for unpaired data. For comparisons among three or more groups, one-way ANOVA with Tukey's multiple comparisons test was applied. The χ^2 test was used to determine the association between KIN17 expression and the clinicopathological variables of the samples. The survival curve was plotted using the Kaplan-Meier method and data were compared using the log-rank test. The relationship

between the expression of two genes was analyzed using Pearson's correlation coefficient. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression of KIN17 in pancreatic cancer and its effects on overall survival rate. Bioinformatics analysis using TCGA database revealed that the expression levels of KIN17 were upregulated in pancreatic cancer tissues compared with those in the adjacent nontumor tissues (Fig. 1A). In addition, the mRNA expression levels of KIN17 were compared between normal and pancreatic cancer tissues in the GSE15471 [expression analysis of 36 pancreatic ductal adenocarcinoma (PDAC) tumor tissues and matching normal pancreatic tissue samples from patients with pancreatic cancer], GSE71989 [8 normal pancreatic from healthy controls and 14 PDAC tissues] and GSE62165 (118 PDAC samples and 13 normal samples from healthy controls) datasets from the GEO database (Fig 1B-D). The expression of KIN17 was significantly upregulated in pancreatic cancer tissues compared with that in normal pancreatic tissues. Additionally, the analysis of microarray slides containing 72 pairs of pancreatic cancer tissues with adjacent tissues revealed that the KIN17 staining intensity was significantly higher in pancreatic cancer tissues than in adjacent tissues (Fig. 1E). Notably, patients with pancreatic cancer and higher KIN17 expression were revealed to have poorer overall survival than those with lower KIN17 expression, as determined by the Kaplan-Meier survival analysis performed on data from 72 patients with pancreatic cancer (Fig. 1F). In addition, a GEO dataset (GSE62452; 69 pancreatic tumors and 61 adjacent non-tumor tissue from patients with PDAC) was used to assess the association between pancreatic cancer survival and KIN17 expression levels; the Kaplan-Meier curves were plotted using the R package survminer. The results showed that patients with pancreatic cancer with high KIN17 expression had significantly lower overall survival than those with low KIN17 expression (Fig. 1G). Clinical data from 72 patients with pancreatic cancer were analyzed. As summarized in Table I, the high expression of KIN17 in pancreatic cancer was positively associated with lymph node metastasis ($P = 0.015$). Tumor-Node-Metastasis stage has been reported in the relevant literature as a risk factor for poor prognosis in patients with pancreatic cancer (21). Subsequently, the mRNA and protein expression levels of KIN17 were detected in normal pancreatic epithelial HPNE cells, and four pancreatic cancer cell lines: ASPC-1, PANC-1, PACA-2 and BxPC-3. The results of RT-qPCR and WB showed that the mRNA and protein expression levels of KIN17 expression were both increased in pancreatic cancer cells compared with those in HPNE cells, but there was no significant difference in KIN17 expression between BxPC-3 and HPNE cells (Fig. 1H and I). These results indicated that KIN17 may be upregulated in pancreatic cancer tissues and cell lines, and that this increase in KIN17 expression is associated with a shorter survival period.

Effect of silencing KIN17 on cell migration and invasion. Before exploring the role of KIN17, Cells were transfected with three siRNAs specifically targeting KIN17 (siRNA #1, #2 and #3) to knock down KIN17 expression, and the most

effective siRNA, siKIN17#3, was selected using RT-qPCR and WB (Fig. S1), which was used in the subsequent studies conducted in PANC-1 and PACA-2 cells. According to our previous studies, using siRNAs to reduce KIN17 expression has shown good knockdown efficiency in hepatocellular carcinoma cells (6,22). In the present study, the siRNA sequence siKIN17#3 had a relatively high knockdown efficiency for KIN17 of ~80%. Wound-healing and Transwell assays were conducted to evaluate the role of KIN17 in migration and invasion. The results indicated that KIN17 knockdown reduced cell migration (Fig. 2A) and invasive ability (Fig. 2B) in PANC-1 and PACA-2 cells. It is widely accepted that EMT makes tumor cells highly mobile and invasive (23). In addition, it has been shown that EMT is involved in pancreatic cancer metastasis (24). The present study revealed that knockdown of KIN17 upregulated the expression of epithelial markers (E-cadherin), and downregulated the expression of mesenchymal markers (N-cadherin and Vimentin) in PANC-1 and PACA-2 cells (Fig. 2C). These results suggested that KIN17 suppression may lead to inhibition of the migration and invasion of pancreatic cancer cells.

Promotion of autophagy by KIN17 knockdown. Previous studies have extensively explored the link between autophagy and tumor metastasis (25,26). The present study assessed the levels of autophagy-related proteins using WB following the knockdown of KIN17 in pancreatic cancer cells. Notably, the depletion of KIN17 in PANC-1 and PACA-2 cells resulted in a marked increase in the expression of autophagy-related proteins, such as Beclin1, LC3II, ULK1 and P-PULK1, and a significant decrease in P62 expression. The possible reason for the simultaneous elevation of ULK1 and P-ULK1 when KIN17 was knocked down may be that when KIN17 is knocked down, pancreatic cancer cells could initiate a compensatory mechanism to increase the expression of downstream proteins to maintain normal physiological functions. In this case, both ULK1 and P-ULK1 may be elevated. Furthermore, phosphorylation is an important protein modification that can change the activity, localization and stability of proteins. When KIN17 is knocked down, the phosphorylation level of the downstream protein ULK1 may change, leading to the elevation of P-ULK1. At the same time, total protein ULK1 may also be elevated for this reason (Fig. 3A). Furthermore, autophagosomes were examined by observing the presence of GFP-LC3 points, revealing a higher number of GFP-LC3 puncta in pancreatic cancer cells transfected with siKIN17 than in siNC cells (Fig. 3B). Next, the mRFP-GFP-LC3 dual fluorescent lentivirus was used to monitor autophagosomes and autolysosomes. After the formation of autolysosomes, their GFP signals are susceptible to acidic conditions, whereas mRFP signals are less affected. Therefore, in the merged figure, yellow dots indicate autophagosomes and red dots indicate autolysosomes (fusion of autophagosomes and lysosomes). The formation of both autophagosomes and autolysosomes increased in PANC-1 and PACA-2 cells transfected with siKIN17, indicating increased autophagic activity compared with in siNC cells (Fig. 4A). These findings suggested that autophagosomes successfully fused with lysosomes instead of being obstructed. In addition, autophagic flux was assessed by monitoring the conversion of LC3I to LC3II. Two autophagy

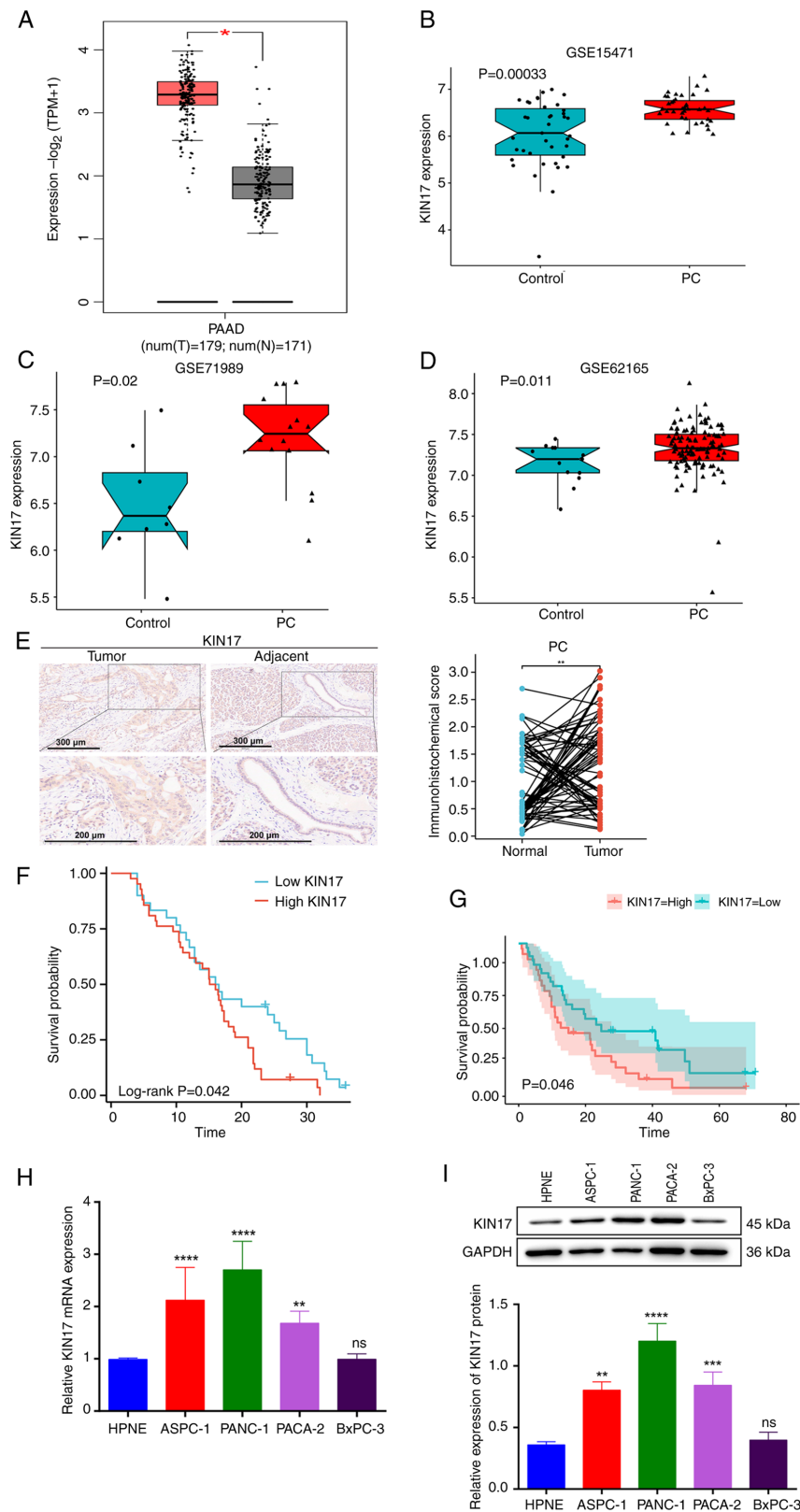


Figure 1. KIN17 is highly expressed in pancreatic cancer and is associated with overall survival. (A) Expression of KIN17 in pancreatic cancer from The Cancer Genome Atlas database. $P < 0.05$. (B) Differential expression of KIN17 in pancreatic ductal adenocarcinoma tumors and matching normal pancreatic tissue samples from patients with pancreatic cancer in the GSE15471 dataset. (C) Differential expression of KIN17 in normal pancreatic and PDAC tissues in the GSE71989 dataset. (D) Differential expression of KIN17 in PDAC samples and control samples in the GSE62165 dataset. (E) Representative KIN17 immunohistochemistry staining images, with semi-quantification in 72 pairs of human pancreatic cancer tissues and adjacent tissues. Scale bars, 300 μm (upper image) and 200 μm (lower images). $P < 0.01$. (F) Analysis and comparison of overall survival rate based on the KIN17 expression level in patients with pancreatic cancer. $**P < 0.01$, $****P < 0.0001$ vs. HPNE. (G) Survival curves for KIN17 levels in patients with pancreatic cancer based on the GEO dataset (GSE62452). (H) Relative mRNA expression levels of KIN17 in HPNE cells, and four different pancreatic cancer cell lines (ASPC-1, PANC-1, PACA-2 and BxPC-3) using reverse transcription-quantitative PCR. $**P < 0.01$, $****P < 0.0001$ vs. HPNE. (I) KIN17 protein levels in HPNE cells, and four different pancreatic cancer cell lines (ASPC-1, PANC-1, PACA-2 and BxPC-3) detected using western blotting. $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$ vs. HPNE. GEO, Gene Expression Omnibus; PAAD/PDAC, pancreatic ductal adenocarcinoma.

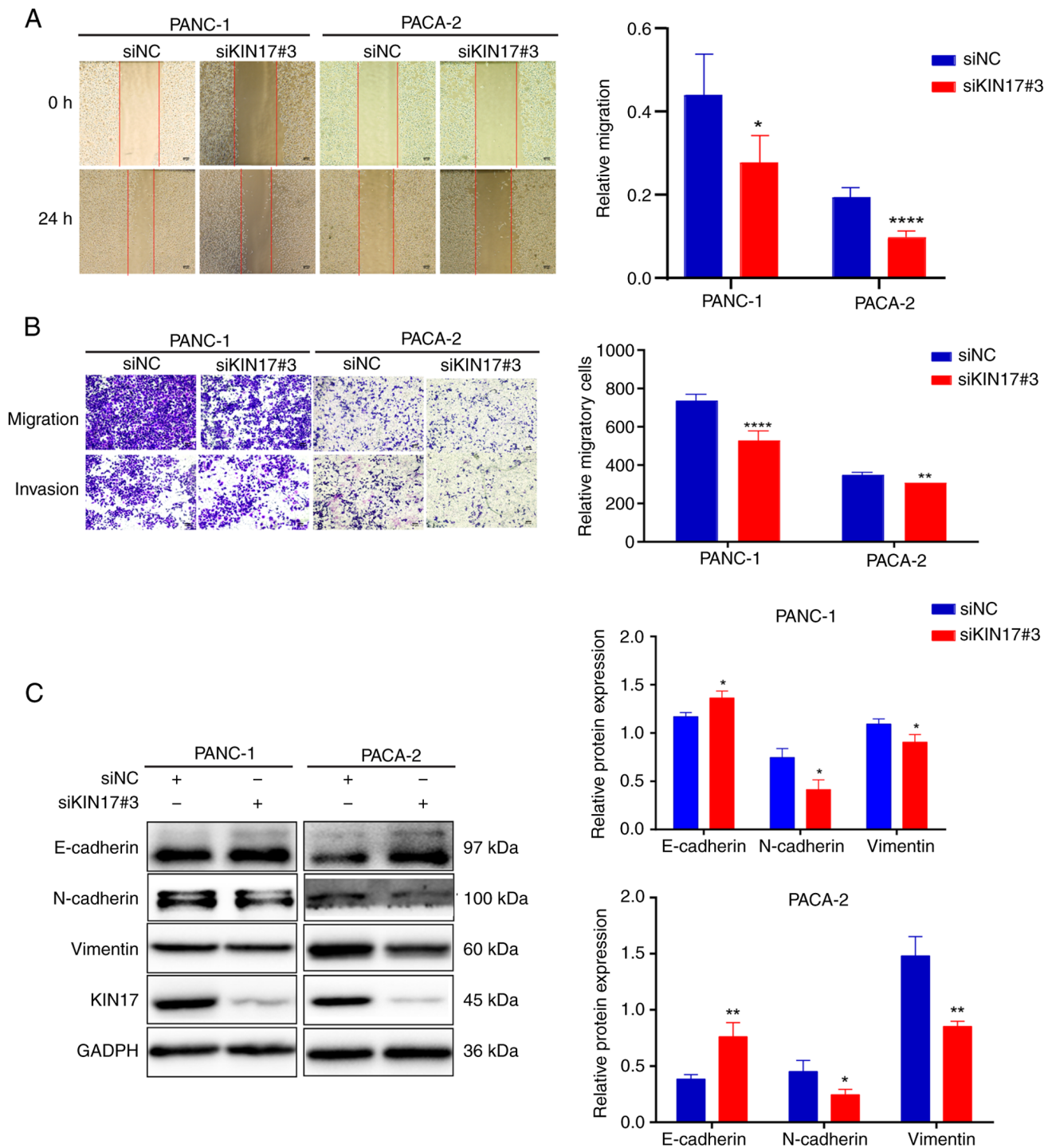


Figure 2. Inhibition of migration and invasion triggered by KIN17 knockdown in pancreatic cancer cells. (A) Migration of PANC-1 and PACA-2 cells in wound-healing assay. (B) Transwell assay for the observation of changes in migration and invasion. (C) Western blotting for the detection of epithelial-mesenchymal transition protein expression in PANC-1 and PACA-2 cells. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$ vs. siNC. NC, negative control; si, small interfering.

inhibitors, 3-MA and CQ, were used in the present study. While 3-MA impedes the formation of autophagosomes in the initial stages by deactivating class III phosphatidylinositol 3-kinase (27), CQ increases lysosomal pH and hinders the fusion of autophagosomes with lysosomes in later stages (28). Comparative analysis revealed that co-treatment with 3-MA significantly decreased KIN17 inhibition-dependent LC3-II protein expression, suggesting a reversal of the autophagic process (Fig. 4B). Conversely, co-treatment with CQ

significantly increased KIN17 inhibition-dependent LC3-II protein expression, indicating that 3-MA and CQ counteracted the autophagy-promoting effects of KIN17 knockdown, this may be due to 3-MA inhibiting autophagosome formation and CQ inhibiting the process of fusion of autophagosomes with lysosomes. Notably, co-treatment with 3-MA and CQ reversed the expression pattern of P62 compared with KIN17 knockdown alone, indicating their ability to attenuate the effect of KIN17 knockdown on P62 degradation in lysosomes.

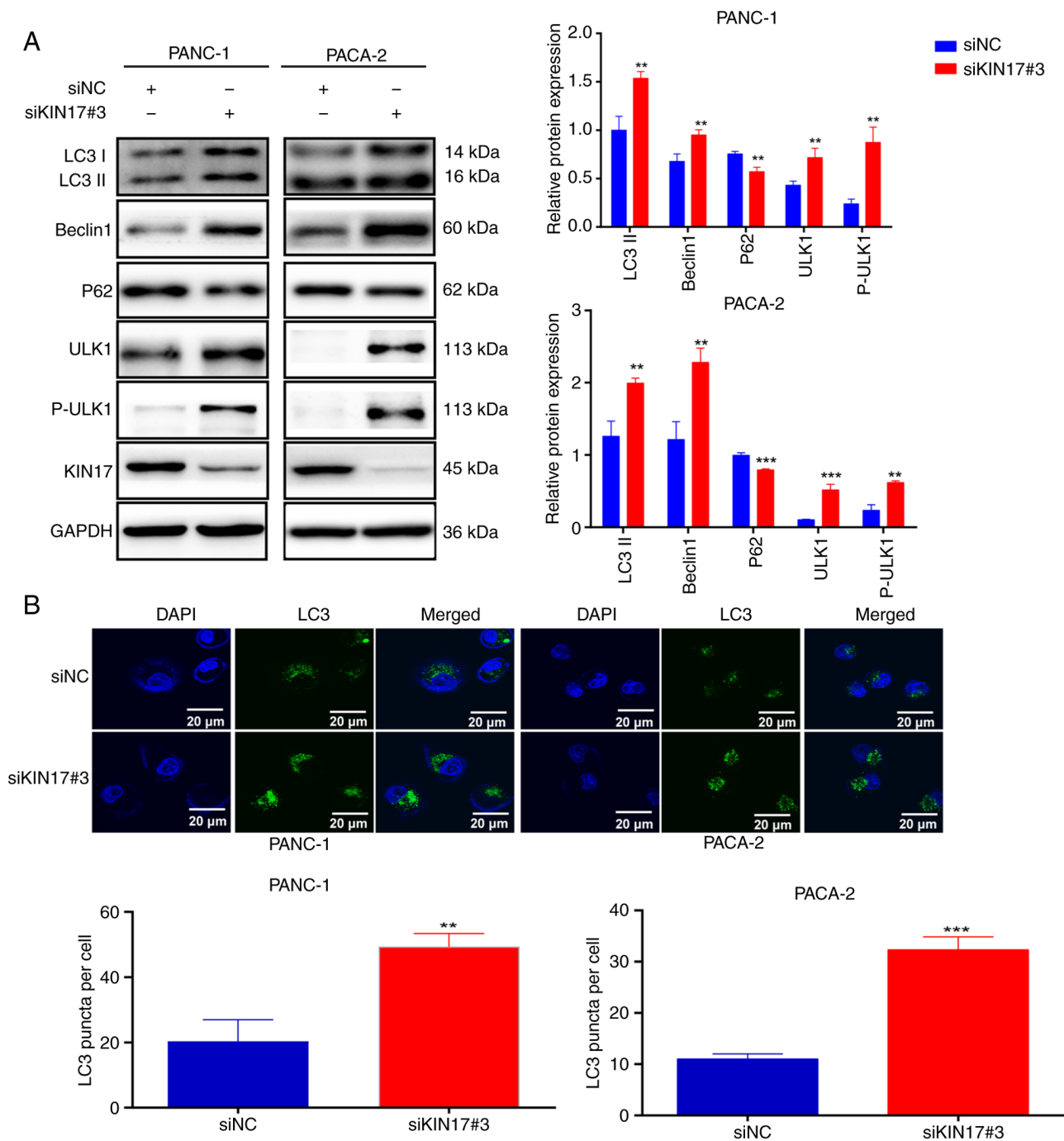


Figure 3. KIN17 knockdown promotes autophagy in pancreatic cancer cells. (A) Western blotting of autophagy-related protein expression in PANC-1 and PACA-2 cells. (B) Representative immunofluorescence images of GFP-LC3 in PANC-1 and PACA-2 cells, and the corresponding percentages of cells containing LC3-II puncta ($n > 15$). Scale bar, 20 μm . ** $P < 0.01$, *** $P < 0.001$ vs. siNC. NC, negative control; P-, phosphorylated; si, small interfering.

These results indicated that KIN17 knockdown may enhance autophagy in pancreatic cancer cells.

Relationship between autophagy and migration/invasion through KIN17 regulation. The induction of autophagy is considered to promote the migratory and invasive capabilities of cancer cells (29). The present study examined the effect of KIN17 inhibition on autophagy induction. Co-treatment of cells with siKIN17#3 and the autophagy inhibitor 3-MA significantly increased migration and invasion of PANC-1 and PACA-2 cells compared with sole treatment with siKIN17#3,

indicating reversal of the anti-migratory and anti-invasive effects mediated by KIN17 knockdown (Fig. 5A and B). These findings suggested that the inhibition of autophagy may counteract the anti-migratory and anti-invasive effects of KIN17 knockdown.

Subsequently, the present study investigated whether simultaneous KIN17 knockdown and induction of autophagy could further enhance the inhibition of migration and invasion in pancreatic cancer cells. Rapamycin, a potent and specific mTOR inhibitor with autophagy-inducing properties, was used in the present study. The results revealed that similar to

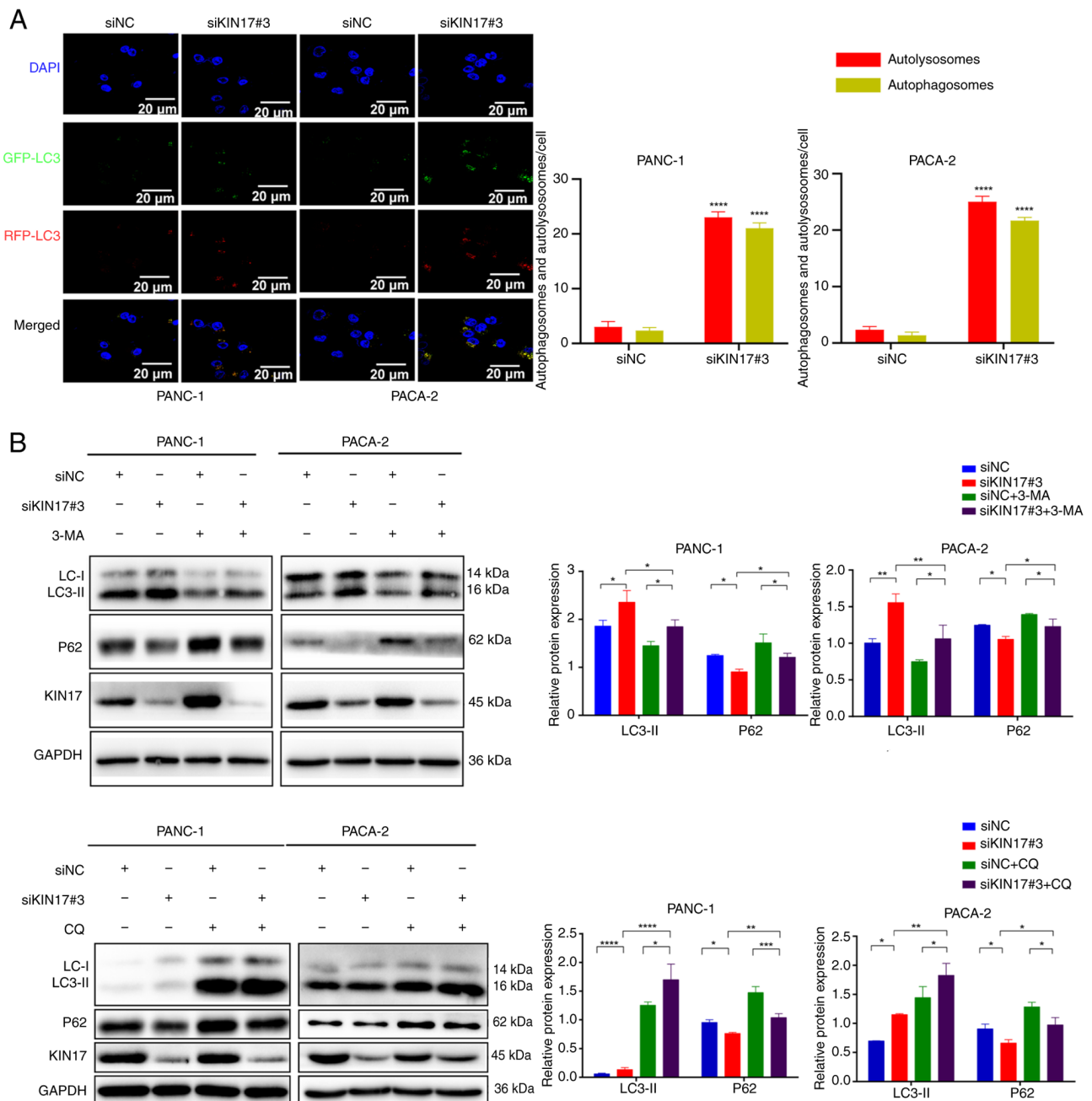


Figure 4. KIN17 knockdown promotes autophagy of pancreatic cancer cells *in vitro*. (A) Representative immunofluorescence images of the mRFP-GFP-LC3 in PANC-1 and PACA-2 cells. Scale bar, 10 μ m. (B) Western blotting for analysis of the effect of siNC/siKIN17#3 combined with autophagy inhibitors [3-MA (2 mM) and CQ (20 μ M)] on LC3-II and P62 protein expression. * P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001. 3-MA, 3-methyladenine; CQ, chloroquine; NC, negative control; si, small interfering.

KIN17 knockdown, siNC + Rapa treatment led to decreased cell migration and invasion compared with siNC alone (Fig. 5C and D). Notably, the combined treatment of Rapa and siKIN17#3 significantly amplified the suppression of cell migration and invasion induced by siKIN17#3. In summary, these results indicated that the combined inhibition of KIN17 and mTOR may effectively boost autophagic activity, leading to synergistic anti-migratory and anti-invasive effects in pancreatic cancer cells.

Role of KIN17 in the PI3K/AKT/mTOR pathway. Increasing evidence has supported the involvement of the

PI3K/AKT/mTOR pathway in autophagy processes (30,31). Through integrated analysis using the GEPIA database, the correlation between KIN17 expression and the PI3K/AKT/mTOR signaling pathway was investigated in pancreatic cancer. The findings revealed a positive correlation between KIN17 expression and AKT and mTOR expression (Fig. 6A-B). Results of WB confirmed that the mTOR, P-mTOR, PI3K, P-PI3K, AKT and P-AKT expression levels were significantly decreased in the PANC-1 and PACA-2 cells with KIN17 knockdown (Fig. 6F). Knockdown of KIN17 decreased both total and phosphorylated proteins of the PI3K/AKT/mTOR pathway, which may be due to a change

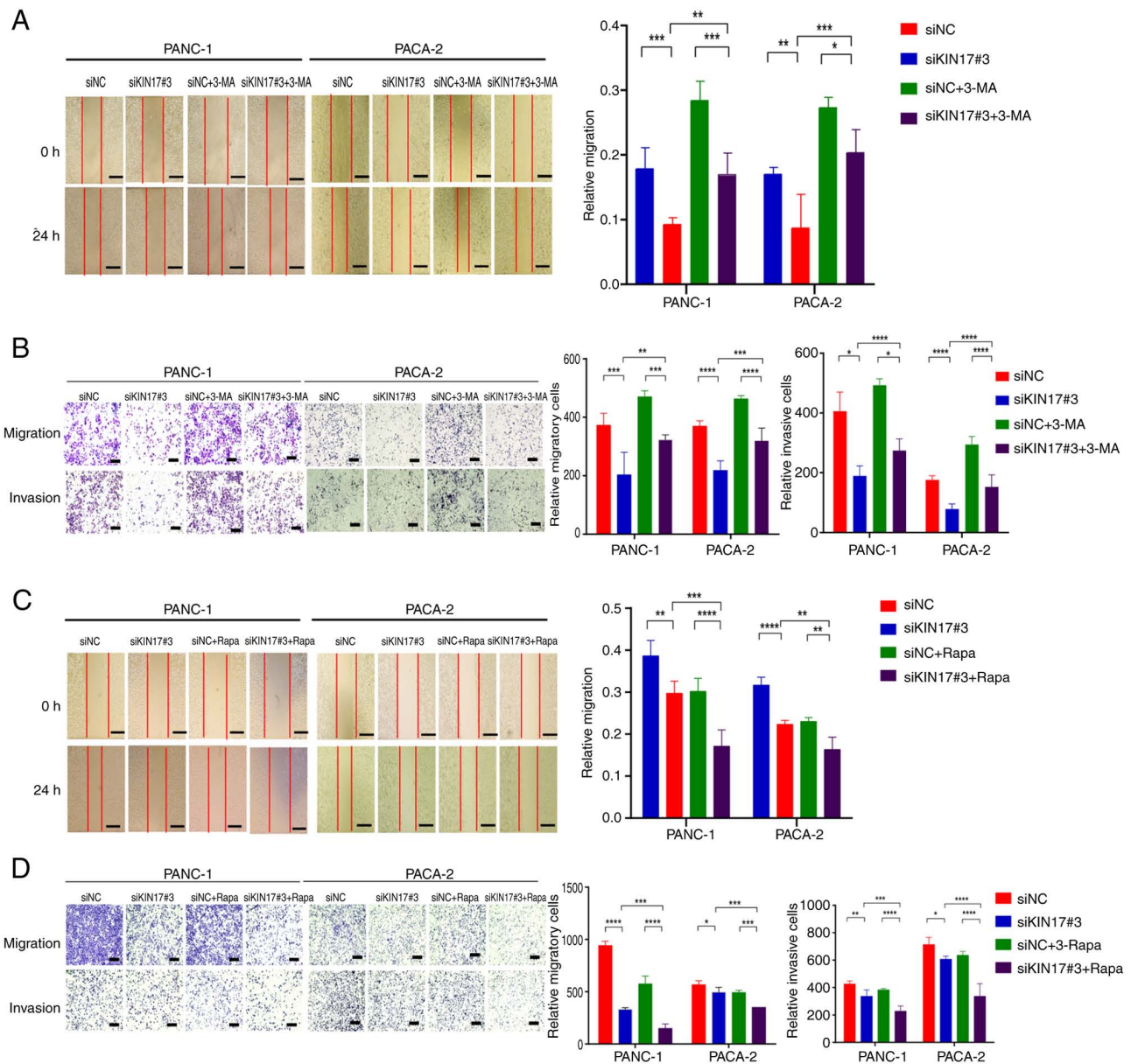


Figure 5. Suppression of KIN17 hampers the migration and invasion of pancreatic cancer cells mediated by autophagy. (A) Evaluation of migration in PANC-1 and PACA-2 cells through a wound-healing assay. Scale bar, 200 μ m. (B) Assessment of migration and invasion in PANC-1 and PACA-2 cells using Transwell chambers. Scale bar, 200 μ m. (C) Analysis of the migration of PANC-1 and PACA-2 cells in a wound-healing assay. Scale bar, 200 μ m. (D) Transwell assay examining the migration and invasion potential of PANC-1 and PACA-2 cells treated with Rapa (100 nM). Scale bar, 200 μ m. * P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001. 3-MA, 3-methyladenine; CQ, chloroquine; NC, negative control; Rapa, rapamycin; si, small interfering.

in protein stability; knockdown of KIN17 may affect the stability of proteins in this pathway, leading to an increase in their degradation, which results in a decrease in the total protein level. Meanwhile, phosphorylation is a key factor in the stability of downstream proteins, thus a decrease in total protein may be accompanied by a decrease in phosphorylated proteins. Taken together, these results suggested that KIN17 may act as a regulatory element within the PI3K/AKT/mTOR pathway, validating the predictions from the GEPIA data.

Discussion

Pancreatic cancer is one of the most aggressive tumors worldwide, which is characterized by early metastasis and a poor prognosis (32,33). Analyzing the mechanisms underlying

early metastasis of pancreatic cancer is of importance in managing its advancement and enhancing patient outcomes. From a clinical perspective, understanding the mechanisms of metastatic progression in pancreatic cancer is crucial for refining current therapeutic approaches. KIN17, a DNA- and RNA-binding protein, is upregulated in various tumors, and its increased expression is associated with unfavorable tumor prognoses, and the invasion and metastasis of cancer cells (5,22). However, research on the role of KIN17 in pancreatic cancer is limited, leaving its biological function and the potential molecular underlying mechanisms unclear. The present study performed a preliminary exploration of KIN17 involvement in pancreatic cancer metastasis, with the aim of identifying a novel target for treating patients with pancreatic cancer.

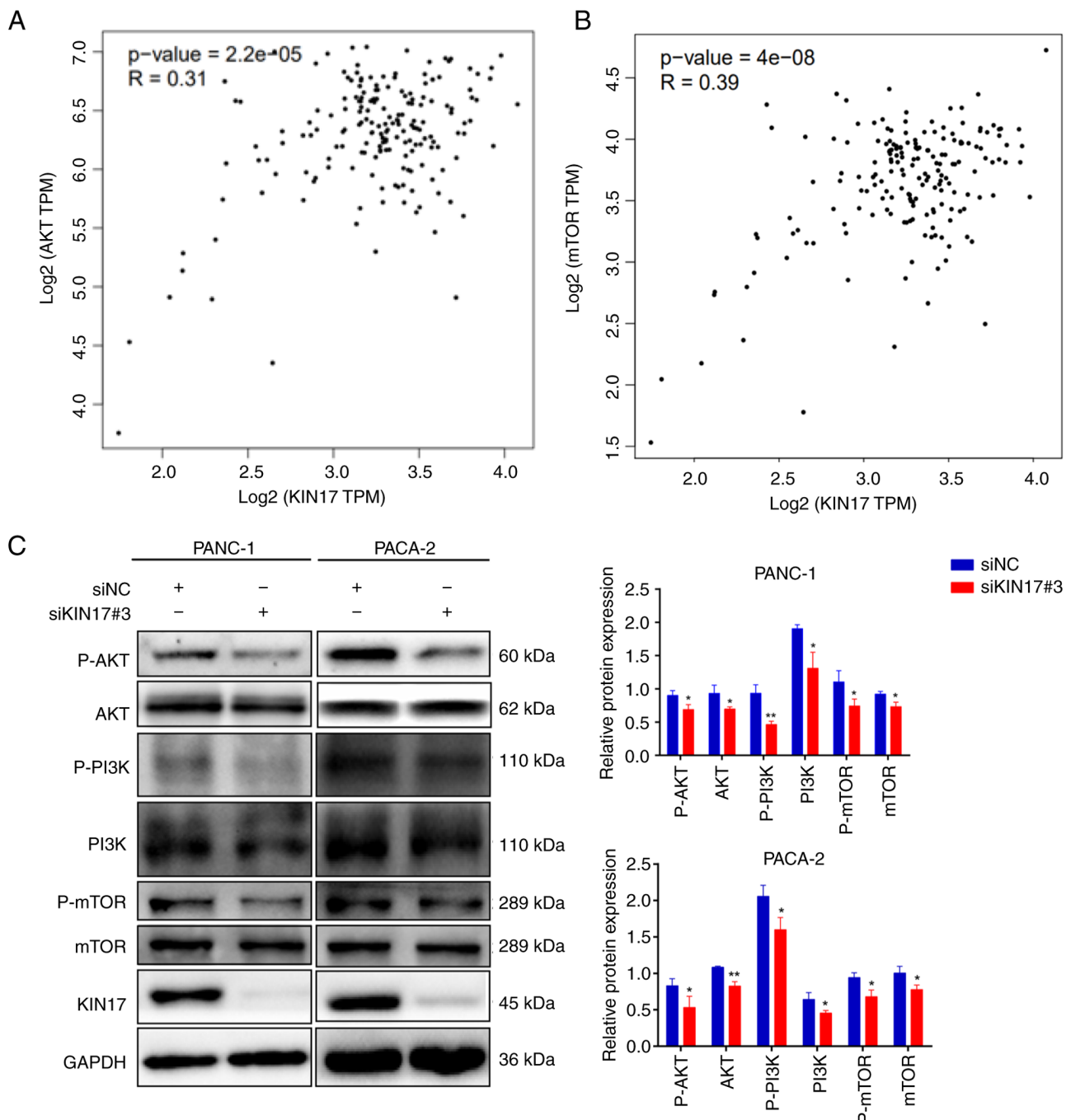


Figure 6. KIN17 is a regulatory factor of the PI3K/Akt/mTOR pathway. mRNA expression of KIN17 was positively correlated with (A) AKT and (B) mTOR, as determined by Pearson's correlation coefficient. (C) Western blotting to detect protein expression in PANC-1 and PACA-2 cells transfected with siNC and siKIN17#3 for 72 h. *P<0.05, **P<0.01 vs. siNC. NC, negative control; P-, phosphorylated; si, small interfering.

The present investigation detected increased KIN17 expression in pancreatic cancer tissue samples compared with that in normal tissues. Using a prognostic model assay, it was demonstrated that increased KIN17 expression was associated with lymph node metastasis in pancreatic cancer. Furthermore, elevated KIN17 levels were associated with a poor prognosis in patients with pancreatic cancer. EMT serves a pivotal role in tumorigenesis and metastasis, and is a physiological process marked by cytoskeletal reorganization and extracellular matrix synthesis. EMT is characterized by the transformation of epithelial cells into stromal cells, facilitating their mobility within the cellular matrix. This transformation involves

reduced intercellular adhesion, loss of polarity, and increased cell motility, invasion and metastasis (34,35). Studies have indicated that EMT induces malignant traits in cancer cells and enhances invasiveness, cancer stem cell activity, and resistance to chemotherapy and immunotherapy (36-40). Increasing evidence has underscored the critical involvement of EMT in the initiation, progression, invasion, migration, metastasis and drug resistance of pancreatic cancer. Therefore, manipulating EMT regulation may emerge as a novel therapeutic approach for the management of pancreatic cancer (41-44). The present results demonstrated that KIN17 inhibition notably upregulated E-cadherin expression, and downregulated N-cadherin

and Vimentin expression in pancreatic cancer cells, thus implying that the inhibition of KIN17 may impede EMT, thereby suppressing pancreatic cancer progression.

Autophagy is a dynamic safeguarding mechanism in which large molecules and organelles are degraded within cells to maintain body equilibrium. Most eukaryotic cells depend on autophagy to regulate the stability of their internal environment. Notably, autophagy serves a protective role in mitigating bodily harm under specific stress conditions, such as ischemia and hypoxia; however, excessive activation of autophagy can trigger programmed cell death, a distinct form of cell death separate from apoptosis and necrosis (45). The role of autophagy in cancer cell death has sparked a debate. While autophagy can sustain intracellular environmental stability by breaking down harmful proteins or damaged organelles and fostering normal cellular metabolism and regeneration, an excessive increase in autophagy can induce autophagic cell death, impede cell proliferation, degrade cancer proteins, thwart tumor initiation, prevent metastasis and even increase chemotherapeutic sensitivity (46). Autophagy and EMT are key biological processes that influence cancer onset and progression, with intricate interconnections between autophagy-related and EMT-related signaling pathways. Previous research has revealed the involvement of autophagy in both inducing and inhibiting EMT, suggesting its potential to stimulate tumor metastasis through EMT induction (47). Conversely, the regulation of autophagy has been documented to prompt molecular shifts from the stromal phenotype to the epithelial phenotype, thereby impeding migration and invasion in various contexts (48). The present study showed that KIN17 knockdown led to an increased expression of classic autophagy markers, such as LC3II, Beclin1 and ULK1, while diminishing P62 levels. These results implied that KIN17 knockdown may induce autophagy. To investigate the impact of autophagy on the migration and invasion of pancreatic cancer cells, Rapa was administered as an autophagy activator, and 3-MA as an autophagy inhibitor to the cells, and the migration of both cell types was subsequently assessed. The observations indicated that the induction of autophagy may curb the migration and invasion of pancreatic cancer cells, whereas autophagy inhibition yielded the opposite outcomes. These results underscore the role of autophagy in regulating EMT in pancreatic cancer cells.

The correlation between KIN17 expression and the mTOR pathway, derived from the GEPIA database, prompted an exploration of the novel role of KIN17 in autophagy modulated by the PI3K/AKT/mTOR pathway (49,50). The PI3K/AKT/mTOR signaling pathway serves as a pivotal metabolic, proliferative and survival regulatory pathway within cells, and is crucial for maintaining cellular equilibrium. This pathway affects tumor development and progression by influencing cellular autophagy in various cancer types, such as laryngeal squamous cell carcinoma, glioblastoma and cervical cancer (51-54). Previous investigations have highlighted the significant role of this pathway in autophagy regulation, from inhibiting autophagy initiation to regulating autophagy processes and terminating autophagy, making it a focal point in autophagy regulation mechanisms (55-58). WB revealed that P-PI3K, P-AKT and P-mTOR levels were decreased following KIN17 knockdown. Previous findings

have confirmed that PI3K/AKT/mTOR is one of the most mutated signaling pathways in human malignancies, including pancreatic cancer, which is activated by various factors such as cytokines, hormones and growth factors (49,50). Nie *et al* (59) reported that ALDH1A3 can activate the PI3K/AKT/mTOR signaling pathway and its downstream target, peroxisome proliferator-activated receptor γ , thus promoting pancreatic cancer metastasis *in vitro* and *in vivo*. Huang *et al* (60) revealed that MSI2 may regulate NLK-mediated EMT and the PI3K/AKT/mTOR pathway to promote pancreatic cancer progression. Likewise, Shao *et al* (61) reported that CPA4 overexpression could contribute to the aggressive clinical stage of patients with pancreatic cancer, and may promote EMT *in vitro* by activating the PI3K/AKT/mTOR signaling pathway. Consistent with the aforementioned findings, the present study suggested that KIN17 knockdown may induce autophagy via the PI3K/AKT/mTOR-mediated signaling pathway in pancreatic cancer cells. However, a single gene can regulate multiple pathways. A limitation of the present study is that other pathways were not considered, which may affect the interpretation of the relationship between KIN17, autophagy and the PI3K/AKT/mTOR pathway. Further analysis is needed to understand this regulatory mechanism in more detail.

In summary, the present study demonstrated that KIN17 inhibition may significantly impede cell migration and invasion by stimulating autophagy and suppressing the PI3K/AKT/mTOR pathway. These results not only shed light on the molecular role of KIN17 in autophagy but also underscore the potential of KIN17 as a promising novel target for the prognosis and treatment of pancreatic cancer. However, the present study has some limitations. First, the mechanism of KIN17 in regulating autophagy should be further studied. Second, the study mainly concentrated on cellular level research and lacks support from animal models or clinical trials, making the research conclusions not comprehensive enough. Further research is needed to remedy these limitations and fully elucidate these issues.

In conclusion, the present study detected high KIN17 expression levels in pancreatic cancer, which were related to lymph node metastasis and poor prognosis. In addition, down-regulation of KIN17 levels was shown to significantly suppress migration and invasion via PI3K/AKT/mTOR signaling-mediated autophagy. These results suggested that KIN17 may be a novel biomarker for pancreatic cancer.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

TZ and QL designed this study and conducted the data analysis, while YY conducted the data search, and extracted and analyzed the data. JT drafted the manuscript and performed data analysis. QL and LTC provided key revisions to the manuscript for important content and interpreted the data. XL provided the technical support and designed study. HC and LC performed clinical data analysis. QL and YY confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The requirement for ethics approval was waived by the ethics committee.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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